

Carbon Balance and Circadian Regulation of Hydrolytic and Phosphorolytic Breakdown of Transitory Starch¹

Sean E. Weise², Stephen M. Schrader³, Kyle R. Kleinbeck, and Thomas D. Sharkey*

Department of Botany, University of Wisconsin, Madison, Wisconsin 53726

Transitory starch is formed in chloroplasts during the day and broken down at night. Transitory starch degradation could be regulated by light, circadian rhythms, or carbon balance. To test the role of these potential regulators, starch breakdown rates and metabolites were measured in bean (*Phaseolus vulgaris*) and Arabidopsis (*Arabidopsis thaliana*) plants. In continuous light, starch and maltose levels oscillated in a circadian manner. Under photorespiratory conditions, transitory starch breakdown occurred in the light faster than at night and glucose-6-P (G6P) was elevated. Nonaqueous fractionation showed that the increase in G6P occurred in the chloroplast. When Arabidopsis plants lacking the plastidic starch phosphorylase enzyme were placed under photorespiratory conditions, G6P levels remained constant, indicating that the increased chloroplastic G6P resulted from phosphorolytic starch degradation. Maltose was increased under photorespiratory conditions in both wild type and plants lacking starch phosphorylase, indicating that regulation of starch breakdown may occur at a point preceding the division of the hydrolytic and phosphorolytic pathways. When bean leaves were held in N₂ to suppress photosynthesis and Suc synthesis without increasing photorespiration, starch breakdown did not occur and maltose and G6P levels remained constant. The redox status of the chloroplasts was found to be oxidized under conditions favoring starch degradation.

In leaves, transitory starch is formed in the chloroplasts during the day and broken down at night. Transitory starch acts as (1) an energy reserve, providing the plant with carbohydrate during the night when sugars cannot be made by photosynthesis, and (2) an overflow, allowing photosynthesis to go faster than Suc synthesis during the day. Transitory starch can be broken down hydrolytically and phosphorolytically (Fig. 1). It is likely that the initial steps of starch degradation are shared between the hydrolytic and phosphorolytic pathways (Fig. 1).

The product of the phosphorolytic pathway is Glc-1-P (G1P), which is normally converted to Glc-6-P (G6P). The plastidic starch phosphorylase cannot use the intact starch granule as substrate and prefers malto-oligosaccharides to larger, branched glucans (Steup and Schächtele, 1981; Shimomura et al., 1982). The products of the hydrolytic pathway, maltose and Glc, are

produced by the action of β -amylase and D-enzyme (DPE1; Smith et al., 2005).

The hydrolytic pathway of starch breakdown has recently been shown to be of primary importance for starch conversion to Suc. Relatively high maltose levels were reported in leaf tissue breaking down starch (Levi and Gibbs, 1976; Peavey et al., 1977; Kruger and ap Rees, 1983; Neuhaus and Schulte, 1996; Servaites and Geiger, 2002). Export studies showed that maltose and Glc were the main exported metabolites from isolated chloroplasts breaking down starch in the dark (Weise et al., 2004). Arabidopsis (*Arabidopsis thaliana*) plants lacking enzymes required for maltose export from the chloroplast and metabolism in the cytosol exhibit a maltose and starch-excess phenotype and stunted growth (Chia et al., 2004; Lu and Sharkey, 2004; Nittylä et al., 2004; Weise et al., 2004). On the other hand, mutants lacking the plastid starch phosphorylase exhibited no phenotype (Zeeman et al., 2004).

Given that hydrolytic starch degradation is the primary pathway for making sugars used for export from the chloroplast at night, we hypothesize that the role of phosphorolytic starch degradation is to supply carbon for metabolism inside the chloroplast. It has been estimated that chloroplasts have sufficient enzymatic capacity to metabolize all the products of starch degradation through the pentose phosphate pathway (Stitt and ap Rees, 1979). The oxidative branch of the pentose phosphate pathway produces NADPH at night and can provide precursors for DNA, RNA, and various phenolic compounds, including the aromatic amino acids. However, this pathway does not produce any products that cannot be made during the day and absence of a phenotype in plants lacking starch phosphorylase activity suggests that, under ambient conditions, the

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² Present address: Department of Metabolic Biology, John Innes Centre, Norwich NR4 7UH, UK.

³ Present address: U.S. Department of Agriculture Photosynthesis Research Unit, University of Illinois, 190 ERML, 1201 W. Gregory, Urbana, IL 61801.

* Corresponding author; e-mail tsharkey@wisc.edu; fax 608-262-7509.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Thomas D. Sharkey (tsharkey@wisc.edu).

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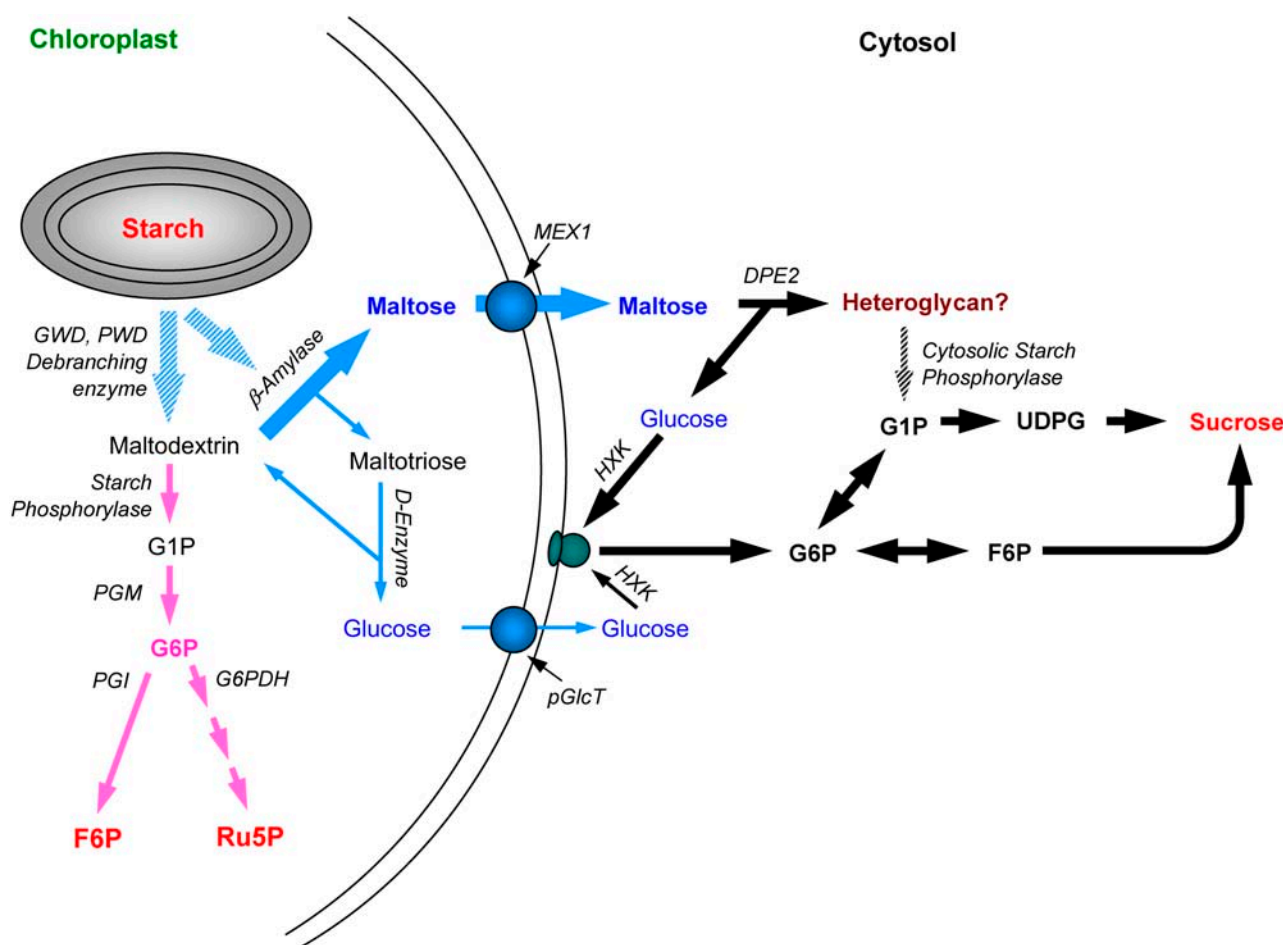


Figure 1. Pathway of hydrolytic and phosphorolytic starch degradation. The products of the hydrolytic pathway are maltose and Glu, which are exported to the cytosol to make Suc. The product of the phosphorolytic pathway is G6P, which is used by the pentose phosphate pathway at night and may be used to regenerate Calvin cycle intermediates during the day. Hatched arrows indicate steps that are currently uncertain. PWD, Phosphoglucan water dikinase; PGI, phosphoglucoisomerase; MEX1, maltose transporter; pGlcT, Glu transporter; DPE2, cytosolic glucanotransferase.

relative importance of the carbon supply from starch to the pentose phosphate pathway may be minor. Under stress conditions, the pentose phosphate pathway may be more important. It was reported that when plants lacking starch phosphorylase activity were transferred from high to low humidity or exposed to salt stress, extensive wilting occurred and necrotic lesions formed on the edges of the leaves. The authors hypothesized that the pentose phosphate pathway is important under stress conditions to control reactive oxygen intermediates by the ascorbate-glutathione cycle and that a limitation of this pathway may result in the observed phenotype (Zeeman et al., 2004).

In most plants, transitory starch is synthesized at a constant rate during the day and broken down at a constant rate during the night. Several factors could be important in regulating starch degradation.

First, light could suppress starch degradation, perhaps by redox control. In plants lacking the triose phosphate translocator, redox control was invoked to explain simultaneous starch synthesis and degrada-

tion (Walters et al., 2004). Recent work by Mikkelsen and coworkers has provided evidence that glucan water dikinase (GWD), which is required for normal transitory starch breakdown, is redox regulated (Mikkelsen et al., 2004). However, GWD was active in the reduced form, inconsistent with a role in restricting starch degradation to oxidizing conditions.

Second, starch degradation could be under circadian control. In C_3 and Crassulacean acid metabolism plants, starch synthesis is under circadian control (Li et al., 1992; Geiger et al., 1995; Borland and Taybi, 2004), and, in a recent study, Lu et al. (2005) showed that, in continuous light, starch degradation in *Arabidopsis* is under circadian control. Transcripts for many of the enzymes involved in hydrolytic starch degradation fluctuate on a diel cycle (Smith et al., 2004; Lu et al., 2005). In continuous light, the transcript levels of many of these enzymes were observed to fluctuate in a circadian manner and the genes for these enzymes contain circadian regulatory elements in their promoters (Lu et al., 2005). However, protein levels

remained relatively constant throughout the day and night (Smith et al., 2004; Lu et al., 2005).

Third, carbon balance could control starch degradation. If one reason for transitory starch synthesis is to supply carbon when photosynthesis cannot occur, we might expect leaf carbon balance to influence starch degradation. Both sugar beet (*Beta vulgaris*) and bean (*Phaseolus vulgaris*) leaves have been found to break down starch at low light early and late in the day (Fondy et al., 1989).

We have tested effects of circadian control and carbon balance of starch breakdown in bean leaves. *Arabidopsis* plants were also used to test the role of plastidic starch phosphorylase. To better understand circadian regulation, starch and maltose levels were monitored in plants placed in continuous light. Photorespiration was used to induce a negative carbon balance in the leaves in the light. Under photorespiratory conditions, levels of starch and metabolites in the phosphorolytic (G6P) and hydrolytic (maltose) pathways were measured and localized. We also investigated starch breakdown in leaves held in N_2 or N_2 with 2% O_2 to suppress photosynthesis and Suc synthesis, but not increase photorespiration. The redox status of the chloroplasts during the day, at night, under photorespiratory conditions, and in 100% N_2 was estimated by measuring the activation state of NADP-malate dehydrogenase (MDH).

RESULTS

Circadian Control of Starch Degradation

Starch and maltose were extracted from bean leaf punches taken at selected time points for 1 d prior to transferring plants into continuous light and for 2.5 d in continuous light. Starch decreased during the night and accumulated the following day, whereas maltose levels were high at night and dropped during the day (Fig. 2). When plants were placed in continuous light, starch levels did not decrease at any time during the continuous photoperiod. However, the net rate of synthesis was almost zero during the time that had previously been the plant's night period (the subjective night; Fig. 2). The starch accumulation rate during the first subjective night in continuous light was significantly less than during either the preceding or the following day, as determined by an ANOVA/Tukey's honestly significant difference (HSD) mean-separation post-test ($P < 0.05$). Maltose levels were low under continuous light but increased some during subjective night (Fig. 2).

To examine starch degradation in response to light/dark signals, the lights were turned off 4 h after the beginning of the day, giving bean plants an early night period. Leaf punches were taken just before the lights were turned off and after 1 and 5 h in darkness. The samples were assayed for G6P, maltose, and starch. When the lights were turned off early, G6P and starch

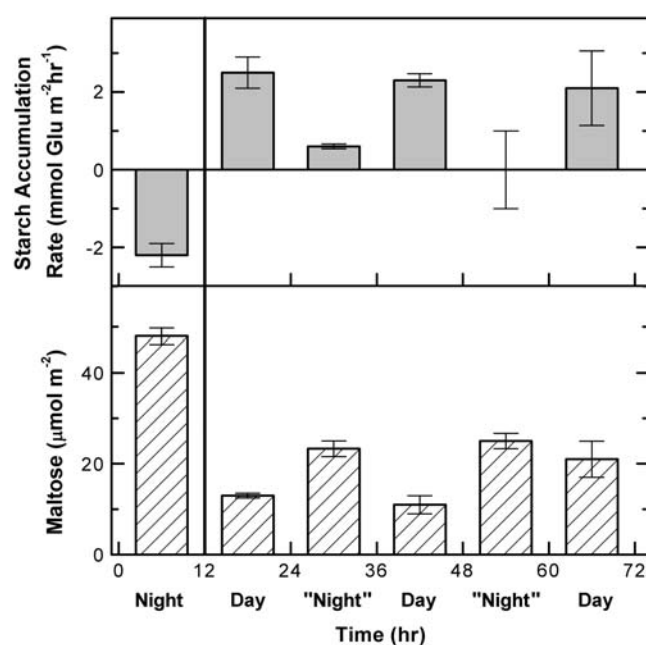


Figure 2. Starch accumulation and maltose under continuous light. During the first 12 h, plants were in the night phase of their normal 12-h photoperiod. After 12 h, represented by a vertical line, plants were in continuous light; night in gray represents subjective night. Whereas no significant decrease in starch levels was observed under continuous light, the rate of accumulation oscillated in a circadian manner. Starch accumulation rates and maltose levels during the first subjective night were significantly different from the previous or subsequent 12 h as determined by an ANOVA/HSD mean-separation test ($P < 0.1$). Values are mean \pm SE; $n = 5$.

levels remained unchanged (Fig. 3). After 1 h in an early night, maltose levels rose by 50%. By comparison, maltose levels were observed to rise 150% after 1 h of normal darkness. Changes in starch levels after 1 h in either night or early night conditions were within the noise of the assay. After 5 h of darkness, starch levels were 40% lower in early night but 70% lower in normal night conditions (Fig. 3).

Effect of Carbon Balance on Starch Breakdown

Under photorespiratory conditions, the starch breakdown rate was $4.5 \pm 1.6 \mu\text{mol carbon m}^{-2} \text{s}^{-1}$, whereas the rate of CO_2 release by photorespiration was $2.1 \pm 0.1 \mu\text{mol carbon m}^{-2} \text{s}^{-1}$ ($n = 5$), revealing that starch breakdown could provide more than enough carbon for photorespiration. Photorespiration was measured for as long as 5 h, resulting in carbon loss far exceeding the amount of carbon in Calvin cycle intermediates, indicating that there had to be a net influx of reduced carbon into the Calvin cycle. It is likely that starch was supplying carbon to the Calvin cycle to support photorespiration.

After 1 h in photorespiratory conditions, G6P and maltose levels increased 3-fold and starch levels decreased 25% (Fig. 3). When starch was breaking down normally at night, G6P levels remained constant,

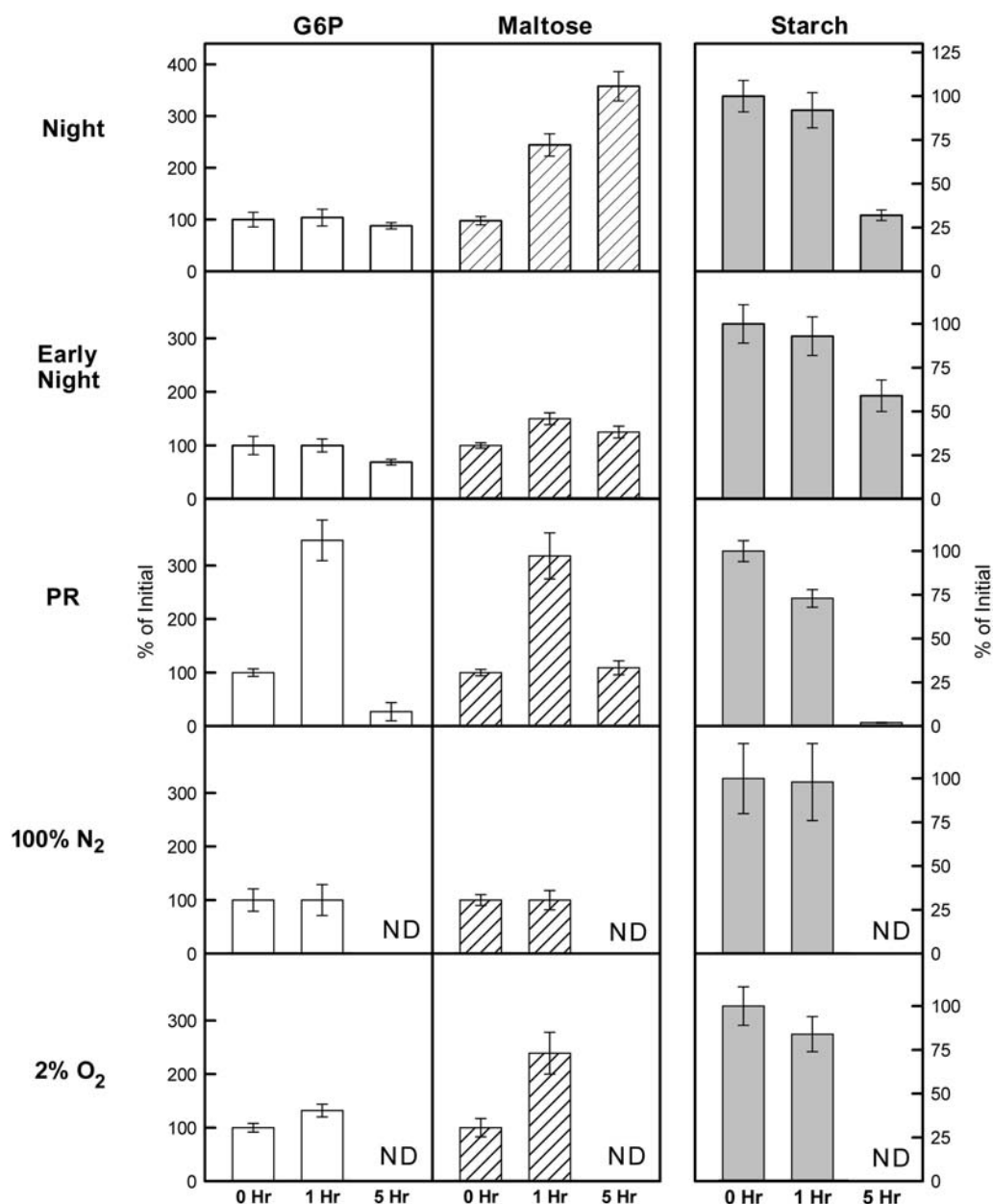


Figure 3. G6P, maltose, and starch levels in bean. Lights were turned off normally 12 h after the beginning of the photoperiod (night) or 5 h after the beginning of the photoperiod (early night). Plants were placed into photorespiratory (PR) conditions, 100% N₂, or N₂ with 2% O₂ 5 h after the beginning of their 12-h photoperiod. 0 h, Immediately before experimental treatment; 1 and 5 h, 1 and 5 h after beginning experimental treatment. ND, Not determined. Values are mean \pm SE; $n = 5$.

maltose levels doubled, whereas changes in starch were within the noise level of the assay (Fig. 3). After 5 h in photorespiratory conditions, almost all the starch was depleted and maltose and G6P levels fell to levels equal to or lower than they were before being placed in photorespiratory conditions (Fig. 3). When bean leaves were placed in 100% N₂ in the light to block the Calvin cycle, the respiration rate was $0.32 \pm 0.06 \mu\text{mol m}^{-2} \text{s}^{-1}$, and G6P, maltose, and starch levels remained constant (Fig. 3). When 2% oxygen was added to nitrogen, the respiration rate increased to $0.68 \pm$

$0.06 \mu\text{mol m}^{-2} \text{s}^{-1}$. A small increase in maltose was observed and G6P and starch levels remained unchanged (Fig. 3).

Evidence for Phosphorolytic Starch Degradation in Vivo

The increase in G6P measured under photorespiratory conditions was primarily in the chloroplast as determined by nonaqueous fractionation of leaves (Fig. 4). G6P levels in the chloroplasts under photorespiratory conditions were more than 3 times the

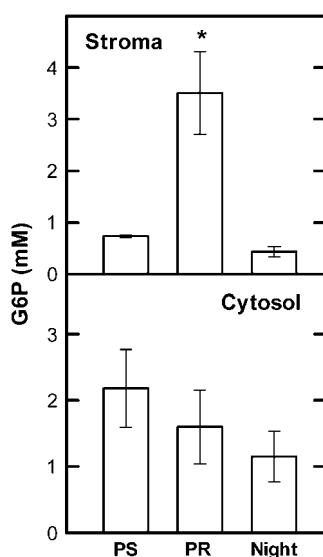


Figure 4. Localization of elevated G6P under photorespiratory conditions. G6P levels in the chloroplast were elevated 3-fold under photorespiratory conditions. G6P levels in the cytosol were relatively constant during all three treatments. Values are mean \pm SE; $n = 3$. Data with an asterisk (*) are significantly different as determined by an ANOVA/HSD mean-separation test ($P < 0.05$). PR, Photorespiration; PS, photosynthesis.

levels measured during photosynthesis or at night (Fig. 4). Wild-type *Arabidopsis* in photorespiratory conditions exhibited a rate of CO_2 release of $2.35 \pm 0.13 \mu\text{mol carbon m}^{-2} \text{s}^{-1}$ ($n = 5$), and G6P and maltose increased, similar to what was observed in bean (Fig. 5). However, when plants lacking plastidic starch phosphorylase (*Atphs1-2*) were placed in photorespiratory conditions, the rate of CO_2 release was $2.35 \pm 0.14 \mu\text{mol carbon m}^{-2} \text{s}^{-1}$ ($n = 5$) and maltose increased, but G6P levels remained constant (Fig. 5). The same result was observed in the *Atphs1-1* line contain-

ing a different mutant allele for plastidic starch phosphorylase (data not shown).

Redox Status of Bean Chloroplasts

Under photorespiratory conditions, the stroma of chloroplasts from bean leaves was significantly oxidized (Fig. 6). NADP-MDH activation was only 37% of activation under photosynthetic conditions. In contrast, under 100% N_2 , the stroma remained fully reduced compared to photosynthetic conditions. In darkness, NADP-MDH activation was not detectable. Total NADP-MDH activities and NAD-MDH activities were equivalent between the different conditions with the mean \pm SE activities at 0.01 ± 0.0005 and $1.6 \pm 0.06 \mu\text{mol m}^{-2} \text{min}^{-1}$, respectively (data not shown).

DISCUSSION

Regulation by Carbon Balance

We found that starch degradation was strongly regulated by carbon balance. When plants were placed in conditions favoring carbon loss by photorespiration, starch degradation proceeded at a rate 3 times faster than the rate at night (Fig. 3). Because the rate of starch breakdown was over twice the rate of CO_2 release, starch degradation could provide more than enough carbon to support photorespiration. Starch breakdown in the light under photorespiratory conditions provides evidence that circadian or light/dark control of the rate of starch degradation can be overridden by internal signals within the chloroplast in response to carbon deficit. Starch breakdown in the light was also found in plants lacking the triose phosphate transporter grown in high light (Häusler et al., 2000; Walters et al., 2004). Presumably, starch breakdown in the light helps plants overcome a

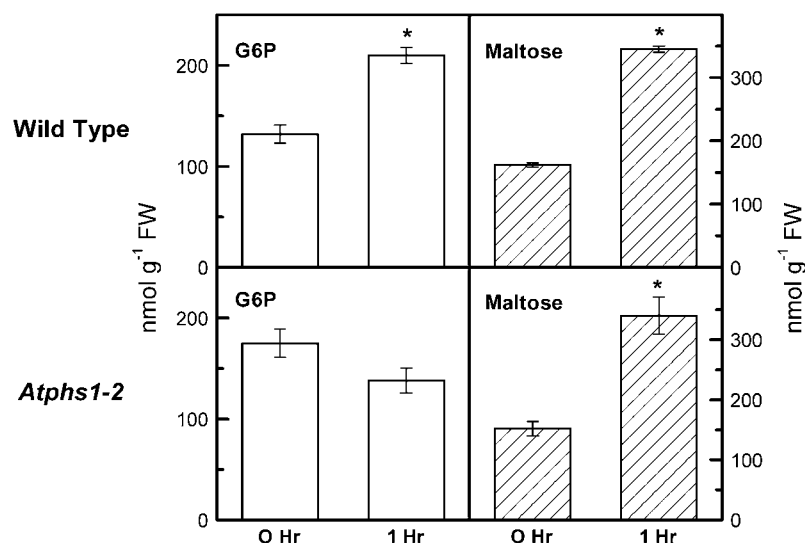


Figure 5. G6P and maltose levels in *Arabidopsis* wild type and mutants lacking starch phosphorylase activity under photorespiratory conditions. In the wild type, G6P and maltose levels were elevated. In the starch phosphorylase-deficient mutant, *Atphs1-2*, G6P levels remained constant or decreased slightly, whereas maltose levels were elevated. Values are mean \pm SE; $n = 5$. Data with an asterisk (*) are significantly different from 0 h as determined by an ANOVA/HSD mean-separation test ($P < 0.05$). 0 h, Immediately before being placed into photorespiratory conditions; 1 h, after 1 h in photorespiratory conditions.

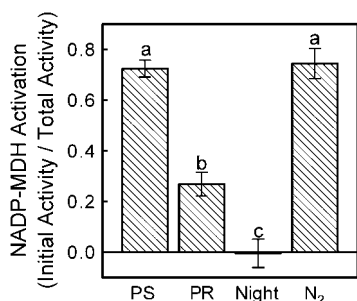


Figure 6. Redox status of the stroma under photosynthetic (PS), photorespiratory (PR), night, and 100% N₂ conditions. NADP-MDH activation was measured after equilibrating the leaf for 1 h under different conditions in a gas exchange cuvette, except for the night-time condition, which was sampled directly from the growth chamber. Values are mean \pm SE; $n = 4$. Data with different letters are significantly different as determined by an ANOVA/HSD mean-separation test ($P < 0.05$).

limitation in triose phosphate export by allowing for export of carbon as maltose. In low light early and late in the day, starch breakdown has been demonstrated in sugar beet and bean (Fondy et al., 1989). Thus, flexibility in the regulation of starch breakdown can overcome both too much carbon in chloroplasts (Walters et al., 2004) and too little carbon in chloroplasts (this article). In both cases, redox control has been suggested as one part of the explanation.

Phosphorolytic Starch Degradation

In photorespiratory conditions, both G6P and maltose increased (Figs. 2 and 5). The increase in G6P was entirely in the chloroplast (Fig. 4) and did not occur in two lines of *Arabidopsis* lacking plastidic starch phosphorylase. We conclude that the increase in G6P results from phosphorolytic starch degradation. This shows that phosphorolytic starch degradation does occur and may be important for supplying carbon for plastid metabolism.

G6P can enter the Calvin cycle through the oxidative branch of the pentose phosphate pathway (Sharkey and Weise, 2006). G6P dehydrogenase (G6PDH) is inhibited by reduction by thioredoxin *m* (Wenderoth et al., 1997). Reduction increases the K_m of the enzyme for G6P but does not eliminate all activity (Scheibe et al., 1989). The low activity of G6PDH in the light is normally important to avoid a futile bypass of G6P to Rib-5-P with the loss of CO₂ (Sharkey and Weise, 2006). G6PDH has a very high K_m when fully reduced, but under photorespiratory conditions the stroma was not fully reduced and the concentration of G6P was very high. This could overcome the high K_m of G6PDH, allowing carbon to flow to Rib-5-P to enter the Calvin cycle.

When bean plants were placed in 100% nitrogen, starch breakdown did not occur, but a low level of starch breakdown was observed in 2% oxygen as evidenced by a slight rise in G6P and a small, but significant, rise in maltose (Fig. 3). We hypothesize that

there was enough photorespiration in 2% oxygen to stimulate a low level of starch degradation. Thus, whenever phosphorolytic starch degradation was stimulated, we also found increased hydrolytic starch degradation. Under 100% N₂ conditions, there would be no Calvin cycle activity and no loss of carbon, but no gain either. It is unclear how the lack of carbon fixation could be distinguished from net loss of carbon. The redox status of the stroma was high during normal photosynthesis and when leaves were in 100% N₂. The highest rate of starch degradation was in photorespiratory conditions when the redox status was intermediate between that during photosynthesis and that found in darkness. The data do not rule out redox regulation but indicate that, if it occurs, it is complex.

Regulation of Starch Degradation

Stimulation of phosphorolytic starch breakdown during photorespiration could be adaptive for providing carbon to keep the Calvin cycle operational. However, the high levels of maltose demonstrate that the hydrolytic pathway of starch degradation was also stimulated. This stimulation of hydrolytic starch degradation is unlikely to result from the lack of carbon flux to Suc because there would be no flux of carbon to Suc when leaves were held in N₂, but no increase in maltose was seen in that condition. The simplest explanation is that regulation of starch degradation in response to carbon balance lies upstream of the divergence in these two pathways. The pathway of starch degradation upstream of β -amylase and starch phosphorylase is still uncertain (Lloyd et al., 2005). Recent work by Mikkelsen et al. (2004) has provided evidence that that phosphorylation of starch by GWD is redox regulated. During the day, GWD is soluble in the stroma in its active, reduced state and becomes bound to the granule at night in its inactive, oxidized state. This, plus the data presented here, indicates that GWD may be required when starch is being laid down because this is when the stroma is reduced. Ritte et al. (2004) showed that the degree of phosphorylation of starch and the rate of phosphate turnover increased during the first 30 min of darkness. This could be the result of activity of the recently described phosphoglucan, water dikinase (Kötting et al., 2005). It will be interesting to determine whether photorespiratory conditions increase the degree of phosphorylation in the same way as darkness (Ritte et al., 2004).

The circadian regulation of hydrolytic starch degradation gave no evidence for simultaneous phosphorolytic starch degradation, but it is difficult to rule out simultaneous phosphorolytic starch breakdown.

CONCLUSION

In conclusion, we have demonstrated that starch breakdown is strongly regulated by carbon status in the chloroplast. Phosphorolytic starch degradation

could be demonstrated during photorespiration. Phosphorolytic starch degradation would be an efficient way to replenish the Calvin cycle with intermediates during photorespiration. Photorespiration also stimulated hydrolytic starch breakdown in the light, showing that both pathways can occur in the presence of light. We hypothesize that regulation of starch degradation by carbon balance occurs at a point preceding division of these two pathways.

MATERIALS AND METHODS

Plant Material and Growing Conditions

For continuous light experiments, bean (*Phaseolus vulgaris* L. cv Linden) plants were grown in two growth rooms at the Biotron facility of the University of Wisconsin. Light in both rooms was provided by a bank of cool-white fluorescent lamps supplemented with incandescent lamps to provide an average photon flux of $400 \mu\text{mol m}^{-2} \text{s}^{-1}$. The temperature was controlled at 24°C when the lights were on and 18°C when the lights were off. Plants were first grown for 3 weeks in a room with a 12-h photoperiod. After 3 weeks of growth, starch and maltose levels were monitored for 1 d by taking leaf punches approximately every 6 h. After 1 d of monitoring starch and maltose, plants were transferred to a room that was under continuous light at 24°C. Leaf punches were taken approximately every 6 h for 3 d under continuous light and starch and maltose levels were assayed.

For early dark experiments, bean plants were grown in a Conviron growth chamber with light provided by cool-white fluorescent lamps supplemented by 12 60-W incandescent lamps providing an irradiance of $400 \mu\text{mol m}^{-2} \text{s}^{-1}$ of light. Plants were grown under a 12-h photoperiod with a day temperature of 24°C, a night temperature of 18°C, and a minimum of 60% relative humidity. Plants were grown for 3 weeks before use in experiments. After 4 h of light, the lights were turned off in the growth chamber. Starch, maltose, and G6P levels were extracted from leaf punches taken just prior to turning the light out (0 h), and 1 and 5 h after the lights had been out.

For photorespiration and 100% N₂ experiments, bean, wild-type Arabidopsis (*Arabidopsis thaliana*), and the starch phosphorylase knockouts (*Atp1s1-1* and *Atp1s1-2*; Zeeman et al., 2004), both of the Wassilewskija ecotype, were used. Bean plants were grown in a Conviron growth chamber with light provided by cool-white fluorescent lamps supplemented by 12 60-W incandescent lamps providing an irradiance of $400 \mu\text{mol m}^{-2} \text{s}^{-1}$ of light. Arabidopsis plants were grown in a Conviron growth chamber with light provided by two 1,000-W metal halide lamps supplemented by six 60-W incandescent lamps. Light was filtered through screening to provide a photon flux density of $150 \mu\text{mol m}^{-2} \text{s}^{-1}$. Both bean and Arabidopsis were grown under a 12-h photoperiod, 60% relative humidity. Arabidopsis plants were grown in UV-stabilized 4.75-in Ray Leach Cone-tainers (Hummert International). These cones have a diameter of 1 in, allowing access to single leaves for gas exchange measurements.

Gas Exchange

Plants were placed in photorespiratory conditions consisting of $0 \mu\text{L L}^{-1}$ CO₂, 30% O₂, 70% N₂, with a leaf temperature of 28°C for 1 h. Photosynthetic conditions consisted of $375 \mu\text{L L}^{-1}$ CO₂, 21% O₂, 79% N₂, with a leaf temperature of 24°C, and N₂ conditions consisted of 100% N₂, with a leaf temperature of 27°C. Light levels were $1,000 \mu\text{mol m}^{-2} \text{s}^{-1}$ for bean and $500 \mu\text{mol m}^{-2} \text{s}^{-1}$ for Arabidopsis. Bean gas exchange was conducted and monitored using a custom lab-built gas exchange system with a LiCor 6262 detector and light from a xenon arc lamp (Tennessen et al., 1994). Arabidopsis gas exchange was conducted using a $0 \mu\text{L L}^{-1}$ CO₂, 30% O₂, 70% N₂ gas premixed from the lab-built gas exchange system described above with a LiCor 6400 standard head and LED light source. Starch, maltose, and G6P levels were extracted from leaf punches taken just prior to placing in the gas exchange cuvette (0 h), and after 1 and 5 h in the gas exchange cuvette.

Metabolite Assays

Metabolite determinations were made using NAD(P)H-linked assays (Lowry and Passonneau, 1972) in a Sigma ZFP 22 dual-wavelength filter-

photometer (Sigma Instrumente). Assays were carried out in phosphate buffer according to Weise et al. (2005). In bean, maltose levels were determined using maltose epimerase (MER) and maltose phosphorylase (MPL) coupled through hexokinase (HXK) and G6PDH according to Weise et al. (2005). The amount of enzyme used for each bean sample was as follows: G6PDH, 1 unit; HXK, 1 unit; MER, 4 units; and MPL, 1 unit. Because of high levels of Glc in Arabidopsis leaves, β -phosphoglucomutase (PGM) was used with MPL and MER coupled through G6PDH to measure maltose. The amount of enzyme used for each Arabidopsis sample was as follows: G6PDH, 1 unit; MER, 4 units; MPL, 1 unit; and β -PGM, 3 units. When using β -PGM, Glc-1,6-bisP was added to the cuvette for a final concentration of $180 \mu\text{M}$. MER, MPL, and β -PGM were obtained from Kikkoman Biochemical (Shirokane and Suzuki, 1995; Shirokane et al., 2000). Because the presence of an unknown contaminant in recent shipments of MPL caused unacceptable drift in the spectrophotometric assays, MPL was filtered through a Centricon centrifugal filter 50,000-MW cutoff (Millipore Corporation) prior to use. All other enzymes and chemicals were purchased from Sigma-Aldrich.

Nonaqueous Fractionation

Nonaqueous fractionation was carried out as described in Weise et al. (2005). A two-compartment analysis method, assuming no G6P in the vacuole, was used (Gerhardt and Heldt, 1984).

MDH Assays

Leaf samples for NADP-MDH activation were taken using a fast-kill apparatus as described by Schrader et al. (2004), except for night-time samples, which were sampled with a hand-held freeze clamp directly from the growth chamber. Leaves were placed in either photorespiratory conditions consisting of $0 \mu\text{L L}^{-1}$ CO₂, 30% O₂, 70% N₂, with a leaf temperature of 28°C, photosynthetic conditions of $375 \mu\text{L L}^{-1}$ CO₂, 21% O₂, 79% N₂, with a leaf temperature of 24°C, or 100% N₂ conditions. Light levels for photorespiratory, photosynthetic, and 100% N₂ were $800 \mu\text{mol m}^{-2} \text{s}^{-1}$. Fully expanded bean leaves were equilibrated for a minimum of 1 h in the gas exchange cuvette under photosynthetic, photorespiratory, or 100% N₂ conditions, after which 8.2 cm^2 of leaf area were rapidly frozen using the fast-kill apparatus while still in the gas exchange cuvette. Leaves under photosynthetic, photorespiratory, and night-time conditions were sampled within the same 24-h period using different leaflets of the same leaf. Leaves under 100% N₂ were measured approximately 1 week later using the same plant but different leaves.

NADP-MDH activation was analyzed as described by Scheibe and Stitt (1988), with the following modifications. Leaf samples were extracted in an ice-cold mortar and pestle with 1 mL of ice-cold extraction buffer (50 mM bicine-NaOH, pH 8.0, 5 mM MgCl₂, 0.5% Triton X-100, 10 mM mannitol, 0.5% polyvinylpyrrolidone, 1% bovine serum albumin, 5 mM dithiothreitol, 1 mM benzamidine, 1 mM ϵ -aminocaproic acid, and 1 mM EDTA) and $10 \mu\text{L}$ of 100 mM phenylmethylsulfonyl fluoride in isopropanol. Phenylmethylsulfonyl fluoride was added just before extraction. Immediately after extraction, leaf samples were centrifuged for 10 s and $50 \mu\text{L}$ of sample were added to a spectrophotometric cuvette containing $810 \mu\text{L}$ of NADP assay buffer (50 mM bicine-NaOH, pH 8.0, 0.12 mM NADPH, 1 mM dithiothreitol, and 1 mM EDTA) to determine the initial activity. The reaction was started by adding $5 \mu\text{L}$ of 400 mM oxaloacetic acid in 500 mM HCl for a final concentration of 2.3 mM oxaloacetic acid. Full activity was measured the same as initial activity, except $100 \mu\text{L}$ of sample were preincubated for 15 min with $40 \mu\text{L}$ of 875 mM bicine-NaOH, pH 9.0, and 500 mM dithiothreitol. NAD-MDH activity was measured for corrections as initial NADP-MDH activity, except $5 \mu\text{L}$ of sample were added to $850 \mu\text{L}$ of NAD assay buffer (47 mM bicine-NaOH, pH 8.0, 0.6 mM NADH, 5 mM MgCl₂, 1 mM EDTA). Extraction and assay buffers were prepared fresh each day with bicine buffer that had been sparged overnight with humidified N₂. Reactions were monitored using a Beckman DU-640 spectrophotometer (Beckman Coulter) measuring the absorbance difference between 340 and 405 nm.

Statistics

Data were analyzed using the program JMP (SAS) using ANOVA and HSD mean-separation test to determine differences.

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